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ENCEPHALOMYELITIS VIRUS MANIFESTED UPON  
MULTIPLICATION IN VECTORS

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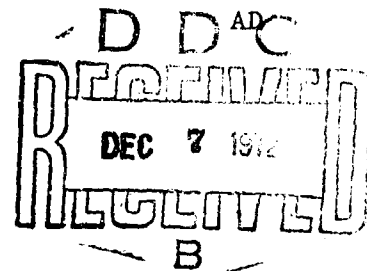
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GENETIC MARKERS OF VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS MANIFESTED UPON MULTIPLICATION IN VECTORS

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A whole series of genetic markers of arboviruses appearing during multiplication in vertebrates (degree of pathogenicity by various means of infection, ability to cause high viremia levels, etc.) has been described in recent times. Arthropod transmission is the active link in the natural cycle of arboviruses. It could be expected that various types of arboviruses would differ according to multiplication in transmission similar to the way they differ according to multiplication in the primary host organism. Absolutely no data in this regard exists in the available literature. We carried out a comparative study in *A. aegypti* mosquitoes of the multiplication peculiarities of clones of Venezuelan Equine Encephalomyelitis (VEE) virus differing in degree of pathogenicity to white mice, plaque size, and certain other genetic markers. *A. aegypti* mosquitoes were not recorded as VEE virus vectors in natural focuses of infection. Nevertheless, they were receptive to the virus under experimental conditions.

Equipment and procedures. The VEE virus clones used in the experiments were partly isolated from natural viral population (3/5, 5, and 17) and partly mutants with mutations induced by lowered temperatures (clones 7 and 14), characterized by residual pathogenicity for adult mice and insignificant virulence for suckling mice. Data on viral properties are given in the table.

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# Peculiarities of multiplication in *Aedes aegypti* mosquitoes of various VEE virus clones and their genetic markers

| No. of clones | Viral accumulation, LD <sub>50</sub> BOE/0.2 ml | Viral antigen in salivary glands | Transmission by bite | Phenotypic appearance of genetic markers |        |        |        |                 |                   |
|---------------|---|----------------------------------|----------------------|--|--------|--------|--------|-----------------|-------------------|
|               |   |                                  |                      | S  | i/c    | i/p    | Vi     | T <sub>60</sub> | ret <sub>40</sub> |
| 3/5           | 5.3   | —                                | —                    | 9.5                                      | 7.6    | 7.8    | 6.3    | 1.0             | —                 |
| 1-7           | 4.5   | —                                | —                    | 3.2                                      | 7.2    | 7.1    | 5.2    | 5.4             | —                 |
| 3-10          | 3.5   | —                                | —                    | 1.7                                      | 5.3    | 3.2    | 1.9    | 6.2             | —                 |
| 1-10          | 3.2   | —                                | —                    | 1.4                                      | 0.5    | 0.5    | 1.0    | 2.5             | —                 |
| 14            | 2.1   | —                                | —                    | 0.2                                      | 0.5    | 0.5    | 0.0    | 6.2             | —                 |
|               |   |                                  |                      | r=0.78                                   | r=0.94 | r=0.96 | r=0.95 | r=0.34          |                   |

The following symbols are used for genetic markers of VEE virus and their phenotypic appearance: S - plaque size in a chicken fibroblast culture. The average diameter is given in millimeters; i/c and i/p - pathogenicity for mongrel white mice weighing 8-9 g. during intracerebral and intraperitoneal inoculation. LD<sub>50</sub> virus titers in 0.03 and 0.2 ml respectively were made. The titer of the tested virus during titration by plaque method was 10<sup>7.0</sup> - 10<sup>7.5</sup> BOE/0.2 ml; Vi - viremia level. A BOE/0.2 ml viral content was put in the plasma. The blood of animals for investigation was chosen according to the appearance of clinical symptoms of experimental encephalomyelitis: T<sub>60</sub> - resistance to heating at 60°. The index of inactivation in lg BOE/0.2 ml after heating for 10 min. was taken. The original viral suspension contained 10<sup>7.0</sup> - 10<sup>7.5</sup> BOE/0.2 ml viruses; ret<sub>40</sub> - ability to multiply at 40°. Plus - virus formed plaques at 40°, minus - plaques not formed.

The *A. aegypti* mosquito culture was obtained from the Institute of Medical Parasitology and Tropical Medicine, Ministry of Health, USSR. The mosquitoes were kept at 27° with a relative humidity of 80-90%.

The accumulation and multiplication dynamics of the viruses in the mosquitoes, the presence of viruses in the salivary glands, and viral transmission by bite were studied.

The mosquitoes were infected by feeding on infected mice or through animal membrane - skin of mouse tail filled with a mixture of virus and defibrinated rabbit or mouse blood in a 1:1 relationship. The viral content in the suspension was 10<sup>6</sup> - 10<sup>7</sup> BOE/0.1 ml.

Quantitative viral determination in the mosquitoes was made by titration of a suspension from the mosquitoes' bodies. From 8 to 15 specimens were included in each test. After washing in physiological solution with antibiotics,

they were put in a porcelain mortar for cooling and ground into a homogeneous mass, then 0.1 ml of physiological solution/mosquito was added. After centrifugation for 15 minutes at 2000 rpm, the supernatant fluid was used for titration by the plaque method, calculating the suspension by the original undiluted material.

Viral antigen was located in the salivary glands by immuno-fluorescence. Acetic liquid immune to VEE virus was used for the tagged antibodies, conjugated with fluorescein isothiocyanate. Viral antigen appeared with the direct method of fluorescence on a background of contrast of nonspecific luminescence of bull albumin tagged by rhodamine sulfofluoride. For staining of the salivary glands the method used by Takaxacu at the National Institute of Health in Tokyo was employed. Isolated salivary glands were fixed on a slide in acetone for 10 minutes, dried, dyed with tagged acetic liquid and contrasted with albumin tagged with rhodamine. During dyeing and contrasting of the preparation with the mixture of conjugated protein (globuline and albumine), it was carefully covered with a cover glass, mixed in a wet chamber, and left 18 hours at 4° for contact. Subsequently, without removing the cover glass, the preparation was carefully washed in a phosphate buffer solution, pH 7.2, by making a layer under the liquid and drawing it off by suction. After washing, the preparation was readied for study in a light microscope at a magnification of 10 x 40.

The coefficients of correlation (r) were determined in accordance with the principles from V. Yu. Urbakh's handbook on biometry.<sup>1</sup>

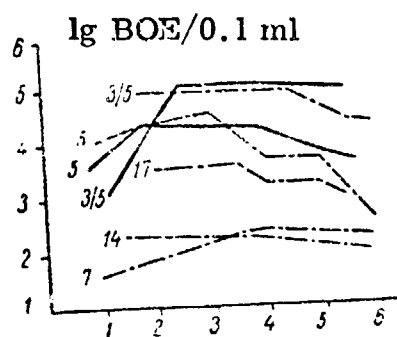
Results. No difference in duration of viral presence in the *A. aegypti* mosquito was observed in the VEE virus clones we studied. In all cases the viruses regularly left the vector after six weeks. Prolonged carrying of the viruses was not accompanied by death of the vector. Regarding the degree of viral accumulation in the mosquitoes, differences among the various clones were noticed (see figure). These differences lasted throughout the experiment.

The degree of viral accumulation in mosquitoes in experiments with clones 3/5 and 5 did not depend on the infection method - whether the mosquitoes were infected by feeding on sick mice or on a viral suspension through an animal membrane (see figure). During work with clones 7, 14, and 17, we used only the latter method, since the given type of virus caused a viremia level in the mice too low to infect the mosquitoes. Viral content in the suspension, which the mosquitoes received by feeding through an animal membrane, and consequently also the infective dose in experiments with clones 7, 14, and 17, was not much less than in experiments with clones actively multiplying in the vector. It may therefore be supposed that the appearance of differences in the degree of viral accumulation is determined by properties of the latter rather than by the method of infection, all the more since the method of infection did not affect viral accumulation in vectors infected by clones 3/5 and 5.

## Dynamics of VEE virus accumulation in *A. aegypti* mosquitoes

Infection of the mosquitoes by feeding on mice - solid line;  
by feeding through animal membrane - dashes. 3/5, 5, 17,  
7 - clone numbers.

Along the ordinate - viral titer. Along the abscissa - time  
of observation (in weeks after infection)



We tried to ascertain the relationship between the ability to multiply in the mosquitoes and certain genetic markers of VEE virus. A precise direct correlation was observed between viral content in the mosquitoes and pathogenicity for white mice during intracerebral and intraperitoneal inoculation, as well as for viremia levels (see table). Despite the small number of observations ( $n=5$ ), the correlation is 99% reliable, in so much as the values found for the coefficient of correlation  $r$  exceed the value  $r_{\min}$  (0.83) during a degree of freedom of  $n=2$ . A positive correlation between the viral content in the mosquitoes and plaque dimensions was 95% reliable, while a negative correlation existed for heat resistance, expressed in indices of viral inactivation, which was statistically insignificant. Viruses that multiplied weakly in the mosquitoes had a  $\text{rct}_{40}$ -marker, while those that multiplied actively were  $\text{rct}_{40}+$ .

Not only quantitative but also qualitative differences were observed between studied clones of VEE virus during multiplication in mosquitoes. In mosquito salivary gland cells infected with actively multiplying clones (3/5 and 5), as well as with clone 17, five to six days after infection viral antigen had been formed. Luminescence of the antigen accumulating in salivary gland cells was bright yellow-green. The control glands, taken from uninfected mosquitoes that contained no specific antigen dyed intense orange-red. Infected mosquitoes transmitted the infection by biting mice. In experiments with clones that multiplied weakly in vectors, in salivary gland cells at various times following infection (4-30 days) viral antigen was not found by immunofluorescence. In biological experiments the virus was not transmitted by bite. Thus, in this case, although the virus multiplied in the mosquito, it did not penetrate the salivary glands.

Discussion. In these tests *A. aegypti* mosquitoes were used as the experimental model for studying the relationship between vectors and VEE virus clones with varying genetic markers. All clones used in the experiment caused the mosquitoes to be carriers for a prolonged period but were not lethal to the mosquitoes. Two types of infective processes were clearly distinguished. In experiments with certain clones active multiplication and high viral accumulation in the vector was observed. Upon reaching the digestive tract, the viruses spread throughout the mosquitoes' bodies, penetrating the salivary glands, accumulating, and being transmitted by bite. In tests with other clones, the virus accumulated in the vector to a significantly smaller degree and did not multiply in the salivary glands. It is possible that viral multiplication was in such cases limited by cells of the digestive tract. Infected mosquitoes did not transmit the virus to receptor animals.

The properties, the genetic constituents, of the virus determined the type of mosquito infection. This fact permits the relationship between vector and genetic markers of the VEE virus to be determined. One can speak of the existence of two extremely closely related genetic markers in evidence during viral multiplication in *A. aegypti* mosquitoes. One of these markers is expressed quantitatively, appearing according to the degree of viral accumulation in the vectors, while the other is expressed qualitatively, in accordance with the ability of the virus to penetrate the salivary glands and be transmitted by bite. These markers, as the methods of statistical analysis show, have a very close direct relationship to markers characterizing viral pathogenicity toward white mice (pathogenicity during intracerebral and intraperitoneal inoculation, viremia level), as well as with markers related to pathogenicity (plaque size, ability to multiply at 40°).

Thus, under experimental conditions, the ability of VEE virus actively to multiply in vectors is combined with high pathogenicity for white mice.

## REFERENCE

1. Urbakh, V. Yu. Biometric Methods (Biometricheskie metody). M., 1964.

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Two genetic markers of Venezuelan equine encephalomyelitis virus manifested upon multiplication of the virus in *Aedes aegypti* mosquitoes and directly related to each other are described: quantitative accumulation of the virus in mosquitoes and the capacity to penetrate into the salivary glands and to be transmitted by bite. These genetic markers are very closely related to genetic markers manifested in virus multiplication in mice: pathogenicity during intracerebral and intraperitoneal inoculation and viremia level.

